PCR Analysis of the Herpesviruses Presence in Crevicular Fluid in HIV- Positive Patients

CRISTINA POPA[1], CARMEN GABRIELA STELEA[1]*, ANA MARIA FILIOREANU[1]*, IRINA GEORGETA SUFARU[1], GEORGE ALEXANDRU MAFTEI[1], MANUELA ARBUNE[2], SILVIA MARTU[1], EUGENIA POPESCU[1]

[1]Grigore T. Popa University of Medicine and Pharmacy, Faculty of Dental Medicine, 16, Universitatii Str.,700115, Iasi, Romania
[2]"Dunarea de Jos University, University of Medicine and Pharmacy, 47 Domneasca Str., 800008,Galati, Romania

The present study aimed to determine the frequency of herpesviruses in gingival fluid samples in patients with periodontitis HIV compared to HIV-negative subjects. Gingival crevicular fluid samples were obtained from 28 patients with HIV-positive periodontitis and from 14 patients with HIV seronegative periodontitis. Herpesviruses have been identified by PCR amplification methods. In HIV-positive patients, the most prevalent herpes virus was HCMV, followed by HHV-6 and HHV-7. In non-HIV-related periodontitis, HCMV was identified in 11 samples and EBV-1 in 8 samples, followed by HSV (7 samples). HIV seropositive samples showed an average of 4.0 herpesviruses and HIV-seronegative individuals averaging 1.4 herpesviruses. EBV-2 and HHV-8 were detected exclusively in subgingival samples from HIV-positive patients. HIV-induced activation of herpes viruses may be a stimulating factor for rapid periodontal destruction. Patients with severe immunosuppression may experience herpesvirus-mediated gingival necrosis. The hypothesis that HIV periodontitis is the result of a combined infection of herpesviruses and bacterial pathogens should be studied further.

Keywords: gingival fluid, PCR, herpesviruses, HIV

The uncertainty about the infectious and clinical events of periodontal disease has given rise to a number of hypotheses regarding the etiology of periodontitis. Some researchers suggest that some infectious agents are the basis for periodontal destruction. Others emphasize the importance of host immune factors and / or genetic features in the occurrence of oral diseases [1-3].

It is assumed that periodontitis begins with genetically predisposed individuals or environmental factors that are infected with virulent infectious agents and exhibit persistent gingival inflammation and distinct immune responses [4-7]. By combining this concept, different herpes viruses have been associated with severe types of periodontal disease [8]. Studies on the viral cause of periodontitis mark an essential point in periodontal research, which until recently was almost exclusively focused on bacterial etiology.

The herpesvirus-bacterial hypothesis of periodontitis proposes that an active herpesvirus infection initiate the destruction of periodontal tissue and that host immune responses to herpesvirus infection are an important component of the disease etiopathogenicity [9].

Herpesvirus infection triggers a release of pro-inflammatory cytokines that have the potential to activate matrix osteoclasts and matrix metalloproteinases and to affect antibacterial immune mechanisms, causing an increase in parodontopathogenic bacteria [10-12]. Herpesviruses and bacterial plaque seem to be able to explain more of the clinical features of periodontitis [8]. The periodontitis risk subject can be managed more efficiently after the acquisition and the means of preventing and treating herpesviruses have been delimited.

Cytomegalovirus infection in neonates and immuno-compromised patients (HIV-infected patients) has a high rate of morbidity. HIV-induced immunosuppression facilitates the reactivation of herpesvirus, but active herpes viruses can also activate latent HIV [13]. The periodontal reactivation of latent herpes viruses by HIV can trigger a cascade of destructive tissue events leading to periodontal destruction. Periodontitis in HIV-infected patients may resemble periodontitis with HIV-negative individuals or be associated with deep gingival bleeding or necrotizing ulcerative periodontitis (NUP) [13].

Cytomegalovirus was identified in 81% of HIV-associated periodontitis lesions and in 50% of non-HIV-associated periodontitis lesions and the herpes virus was most commonly identified. In HIV infected individuals, cytomegalovirus was also involved in acute periodontitis, in the formation of periodontal abscess, in mandibular osteomyelitis and in chronic refractory sinusitis. Virions were detected by SEM methods (electronic microscopy) in 56% of gingival tissues from HIV-positive patients with NUP [9].

Epstein-Barr type 1 virus was more commonly identified in subgingival sites of HIV-positive patients than in subgingival sites of HIV-negative patients (72 vs. 48%) [14]. The Epstein-Barr type 2 virus, which is common in HIV-infected subjects, was detected in 57% of the biopsies of HIV-associated periodontitis lesions but was absent in non-HIV-associated periodontitis biopsies [15]. Human Herpesvirus-8 was present in periodontal lesions in 24% of HIV-infected individuals without clinical signs of Kaposi's sarcoma but was not detected in periodontitis sites of non-HIV-infected individuals [16].

Herpes simplex virus, Epstein-Barr virus, cytomegalovirus and human herpesvirus genome 8 commonly occur in the saliva of HIV-infected persons and have been linked to oral ulcerous lesions, widespread gingival and mucosal inflammation, and oral cancer [17].

Necrotizing ulcerative gingivitis (NUG) or necrotizing ulcerative periodontitis (NUP) affects immuno-compromised, undernourished and psychosocial stressed individuals. In Europe and the United States, NUG and NUP develop primarily in adolescents and young adults, especially HIV-infected people and almost never in young children. In developing countries, NUG can extend

* email: carmenstelea@yahoo.com; afilioreanu@yahoo.com

considerably beyond periodontium and can cause a life-threatening disease called noma or cancrum oris. Noma primarily affects children and is sometimes preceded by a viral infection, such as herpes gingivitis or measles or HIV [18], which may affect the host's defence against resident viruses and pathogenic bacteria. Untreated oral diseases have high mortality rates in developing countries due to a high burden of immunological disease and limited access to professional diagnosis and treatment.

Cytomegalovirus has demonstrated a necrotising potential in acute retinal necrosis in subjects with severe immunosuppression, acute necrotizing esophagitis, necrotizing enterocolitis of preterm infants, necrotizing glomerulonephritis of renal transplantation, necrotizing myelitis and necrotizing encephalitis [9].

Acute infection with the herpes zoster virus resembles NUG and causes osteonecrosis and spontaneous exfoliation of teeth in the region innervated by the affected trigeminal nerve [8]. Infectious agents also participate in the type of osteonecrosis of the jaw which is caused by the use bisphosphonates for the treatment of osteoporosis, bone complications of cancer, malignant hypercalcaemia and Paget's disease [19-21]. A dental infection can induce necrotizing fasciitis with extensive necrosis in subcutaneous tissue and fascia and a high mortality rate. The extent to which cytomegalovirus or other herpesviruses are involved in necrotizing oral cavity is an important subject for research.

The present study aimed to determine the frequency of 7 herpesviruses in gingival fluid samples in patients with HIV periodontitis. Herpesviruses have been identified by PCR amplification methods.

Experimental part

Gingival crevicular fluid samples were obtained from 28 patients with HIV-positive periodontitis (15 male subjects and 13 female patients between 29 and 62 years) and from 14 patients with HIV seronegative periodontitis (8 males and 6 women between the ages of 27 and 64).

The crevicular fluid was taken from representative sites (periodontal pouches deeper than 4mm) as follows: before sampling, the tooth was isolated with cotton rolls, the supragingival bacterial plaque was carefully removed and the site was gently dried with the spray of air. A sterile paper cone (Dentsply Maillefer, Tulsa, OK, USA) was inserted into each selected periodontal pocket, left in place for 30 s and then immediately inserted into sterile Eppendorf tubes which were stored at -20°C to further analysis. In case of visible contamination with blood, the paper cone has been removed and a new site has been selected. The paper cones were thawed, cut to 1 cm in length and thawed with 50 ìl 1X [13 mM Na2 HPO4, 7 mM NaHPO4, 100 mM NaCl (*p*H 7.0)] buffer at 4°C.

A nested PCR methodology was used to detect viral DNA from HIV, HCMV, EBV-1 and EBV-2, HSV, HHV-6, HHV-7 and HHV-8. The outer and inner oligonucleotide primer sets, as well as the sensitivity and specificity of the PCR reactions, were used. Briefly, each PCR test tube contained 25-100 mL of the total volume, which included 2-5 mL DNA template, 20-50 pmol of a pair of outer primers, MgCl 2 at different concentrations, 0.2 mM of each deoxynucleoside triphosphate (dATP, dCTP, dTTP, dGTP), 1.25 U Thermus aquaticus (Taq) polymerase, 10X Taq buffer and double distilled water. The preparation was overlaid with two drops of mineral oil (Sigma Chemical Co.).

The PCR detection limit was determined using diluted viral DNA positive controls. The amplicons for all PCR reactions were detected by electrophoresis at 4 V / cm in Tris-Acetate-EDTA buffer from a 15-20 mL sample in a 2% agarose gel (Life Technologies, Gibco BRL, Gaithersburg, MD) containing 0.5 mg / ml of ethidium bromide. A 1 kb and 50 bb scale digest (Life Technologies, Gibco BRL) served as molecular size markers in electrophoresis assays. The electrophoretic DNA bands were visualized under 300 nm ultraviolet light.

The Chi square test was used to compare the frequency of herpesvirus detection in samples associated with HIV periodontitis and those not associated with HIV. The t Student test was used to compare the number of herpes viruses in periodontal lesions of HIV and non-HIV patients. A p value of less than 0.05 was considered statistically significant.

Results and discussions

21 out of 28 (75%) HIV-positive patients presented genomic HIV sequences in gingival samples. In HIV-positive patients, the most prevalent herpes virus was HCMV, detected in 18 samples, followed by HHV-6 and HHV-7 each detected in 17 samples. In non-HIV-related periodontitis, HCMV was identified in 11 samples and EBV-1 in 8 samples, followed by HSV (7 samples). Table 2 shows the frequency of 7 herpesviruses studied in HIV and non-HIV periodontitis lesions.

HIV seropositive samples showed an average of 4.0 herpesviruses and HIV-seronegative individuals averaging 1.4 herpesviruses.

Interestingly, EBV-2 and HHV-8 were detected exclusively in subgingival samples from HIV-positive patients.

Current treatment for HIV infection consists of HAART (very active antiretroviral therapy), which includes a cocktail of at least three drugs in at least two classes of antiretroviral drugs: typically, two analogous nucleoside analogue reverse transcriptase inhibitors together with either protease inhibitor or a non-nucleoside reverse transcriptase inhibitor [22]. An approved HIV drug class, known as CCR5 antagonists, blocks the CCR5 co-receptor and is prescribed to patients who are infected with several antiretroviral agents resistant to HIV-1 R5 strains.

The vast majority of recurrent or secondary infections of herpes simplex virus manifest itself in the oral-facial area. Vaccines with herpes simplex virus can be very

Table 1
DEMOGRAPHIC PARAMETERS OF THE PATIENTS INCLUDED IN THE STUDY

Parameter	Study group (HIV-positive patients) (n = 28)	Control group (HIV-negative patients) (n = 14)
Age (mean; interval)	43.65 years; 29-62 years	41.43 years; 27-64 years
Gender (Male/Female)	15/13	8/6
Environment (Urban/Rural)	19/9	9/5
Timeframe from the HIV diagnosis (mean; interval)	11.41 years (6-19 years)	-

Table 2
PRESENCE OF HERPESVIRUSES IN STUDY PATIENTS

Herpetic virus	Study group (HIV-positive patients) (n = 28)	Control group (HIV-negative patients) (n = 14)	p Value
HSV	10 (35.71%)	7 (50%)	>0.05
EBV-1	15 (53,57%)	8 (57.14%)	>0.05
EBV-2	15 (53,57%)	0 (0%)	<0.05
HCMV	18 (64,28%)	11 (78.57%)	>0.05
HHV-6	17 (60.71%)	4 (28.57%)	<0.05
HHV-7	17 (60.71%)	3 (21.42%)	<0.05
HHV-8	4 (14.28%)	0 (0%)	>0.05

prevalent in immunocompromised or severely impaired individuals, including children with oncological diseases and in renal transplant patients [23]. Herpes simplex virus can also play a role in radiation-induced oral mucositis.

Recurrent herpes infections can be triggered by stimuli such as fever, stress, cold, menstruation, and ultraviolet radiation. Prematomic symptoms, including paraesthesia, sensitivity, pain, burning sensation, tingling sensation or itching occur at 46-60% of the patients and last for about 6 hours [9].

Necrotizing ulcerative gingivitis (NUG) is characterized by gingival papilla necrosis, bleeding, pain and occasionally fever. In immunocompromised severely impaired patients, NUG may progress to necrotizing ulcerative periodontitis or necrotizing ulcerative stomatitis and to potentially fatal disease called noma or cancrum oris. NUG and progressive disease variants are commonly found in HIV-infected patients and in severely malnourished individuals in developing countries. Jimenez et al. [24] described 45 children without HIV and young adults from Colombia with severe GUN or noma. All patients were from low socio-economic groups with potential predisposing factors such as acute herpetic gingivostomatitis, measles and acute lymphoblastic leukaemia. Malnutrition and reduced oral hygiene favoured the process of necrosis and progression of gum disease to deeper and deeper facial tissues.

At the onset of the AIDS epidemic, periodontal disease was described as one of the oral manifestations of HIV infection. Immunosuppressed patients have a rapid onset and accelerated disease progression depending on their immune status. Periodontitis is an infectious disease involving specific bacteria and characteristic humoral and cellular responses of the host [25]. Connective tissue of inflamed periodontal sites reveals a dense infiltration of mononuclear cells, especially T lymphocytes, B lymphocytes and macrophages [26].

HIV-induced immunosuppression is known to facilitate the reactivation or re-infection of herpesviruses. In HIV patients, concomitant EBV / HCMV and HSV / HCMV infections also increase the potential for reactivation of the herpes virus. Active herpes virus infection may be partly responsible for increased crevicular level of interleukin-1b and tumour necrosis factor associated with HIV periodontitis [27]. In addition, herpes viruses could interact with HIV at the cellular and molecular level to increase the rate of periodontal tissue destruction.

This study detected EBV-2 in gingival fluid from periodontitis lesions with HIV, but not in gingival tissue from non-HIV-related periodontitis. Detection of EBV-2 in HIV periodontitis correlates well with other studies showing an increased frequency of EBV-2 in HIV-infected individuals [15]. EBV-1 and EBV-2 differ in geographical distribution and EBV-2 causes a higher B lymphocyte in vitro than EBV-1 [28].

The present study also detected HHV-6 with a higher frequency in HIV patients than in non-HIV patients. The potential for HHV-6 to induce periodontal disease requires further study. HHV-8 was detected exclusively in subgingival samples of HIV-positive patients.

Perhaps due to the low number of study samples, HCMV, EBV-1, HSV, HHV-7 and HHV-8 were each detected with similar frequency for HIV-associated and non-HIV periodontal lesions. However, HIV periodontal lesions have shown significantly more co-infections with herpesvirus than periodontitis lesions in non-HIV patients. Co-infections with the herpes virus can cause particularly severe immunosuppression that could trigger the proliferation of parodontopathic bacteria and other pathological events associated with destructive periodontal disease.

Oral HIV-induced manifestations of herpes viruses are reported frequently [29]. In immunocompromised patients, EBV has been implicated in the etiology of oral periodontal leukoplasia, Hodgkin's disease, peripheral T-cell lymphoma, and B-cell lymphoproliferative diseases [29]. HCMV infection in these patients may cause painful ulceration and erosions in the lips, tongue and mucous membranes, usually co-infected with EBV.The emergence of high-activity antiretroviral therapy (HAART) reduced the prevalence of oral lesions, especially HIV-related periodontal disease and the incidence of opportunistic HIV disease [30,31].

Briefly, herpesvirus infections during HIV periodontal period may enhance local immune suppression, affect periodontal protective immunity, induce proinflammatory cytokine production, alter the structural integrity of periodontitis, and lead to overgrowth of periodontal bacteria.

Conclusions

The present study suggests that HIV periodontitis may be the result of mixed infection between HIV-herpesvirus-parodontopathogenic bacteria.

HIV-induced activation of herpes viruses may be a stimulating factor for rapid periodontal destruction. Patients with severe immunosuppression may experience herpesvirus-mediated gingival necrosis. The hypothesis that HIV periodontitis is the result of a combined infection of herpesviruses and bacterial pathogens should be studied further.

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